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Crocin inhibits RANKL-induced osteoclast formation and bone resorption by suppressing NF-κB signaling pathway activation

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ABSTRACT

Crocin is a dietary compound with antioxidant and anti-inflammatory properties, but its effects on bone resorption have not been well characterized. Here we address this issue by examining the direct effects of crocin on osteoclast cells *in vitro*. Osteoclastogenesis was induced by RANKL (receptor activator of NF-κB ligand) in mouse bone marrow-derived macrophages in the absence or presence of crocin at various concentrations. Further, the bone resorption activity of mature osteoclast treated with crocin was assessed by pit assay. Without altering cell viability, crocin was shown to inhibit the differentiation and function of osteoclast cells in a dose-dependent manner. Mechanistically, RANKL-induced NF-κB and NFATc1 activation, the critical signaling pathways for osteoclast differentiation and function, were both repressed by crocin in bone marrow-derived macrophages. Thus, crocin suppresses osteoclastogenesis through direct inhibition of intracellular molecular pathways, which may contribute to future development of anti-bone resorption treatment.

1. Introduction

Enhanced bone resorption, caused by pathological activation of osteoclast cells, is a key driver of bone loss-associated diseases, such as osteoporosis, arthritis, osteomyelitis, periodontal bone loss and Paget's disease (Boyle et al., 2003; Redlich and Smolen, 2012). In these situations, patients usually suffer from an imbalance between less bone formation (from osteogenic formation) and more bone resorption (from osteoclast differentiation), resulting in a disruption of the maintenance of physiological bone mass (Hofbauer and Schoppet, 2004; Redlich and Smolen, 2012; Walsh and Gravallese, 2010). In addition to regeneration of sustainable bone tissue through osteogenesis (Goldring and Goldring, 2007; Redlich and Smolen, 2012), blocking bone resorption (Hofbauer and Schoppet, 2004; Jimi et al., 2004; Romas et al., 2002) is often a therapeutic choice. In fact, for treating diseases that are associated with severe bone loss, a series of anti-osteoclast agents have been developed such as bisphosphonates (McClung et al., 2013). Nevertheless, their use has some limitations because of the serious adverse effects associated with these drugs (Tanvetyanon and Stiff, 2006). Moreover, oxidative stress and chronic inflammation, as critical mediators linked to the activation of bone resorption, can stimulate strong systemic effects as well as local responses in activating osteoclast (Aloe et al., 1993; Edwards et al., 2006; Redlich et al., 2002). In this sense, a potential treatment for inflammatory bone loss is the simultaneous inhibition of inflammation and oxidative stress. For instance, crocin, a natural compound containing antioxidant and anti-inflammatory properties (Alavizadeh and Hosseinzadeh, 2014), has been shown to attenuate or prevent bone deterioration in animal models of arthritis or osteoporosis (Cao et al., 2014; Hemshekhar et al., 2012). However, the direct influence of crocin on osteoclast has not been investigated.

Osteoclast formation needs a specific activation of RANKL/RANK (Receptor activator of NF-κB ligand and its receptor) system in macrophage/monocyte precursors (Boyle et al., 2003). Following cell differentiation, RANKL continues to promote the activity of mature osteoclast by stimulating structural changes that enable cell to resorb bone, including cytoskeleton rearrangements, sealed compartment formation next to the bone surface and secretory components of bone-degrading enzymes (Boyle et al., 2003; Wada

Abbreviations: RANKL, receptor activator of NF-κB ligand; BMMs, bone marrow-derived macrophages.

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et al., 2006). To activate these functions, RANKL must initiate several downstream signaling cascades. NF-κB (Nuclear factor-κB) pathway is one of the well-studied pathways of RANKL activation in osteoclast, which is rapidly stimulated following ligand binding on the cell surface. Together with activator protein-1 (AP-1), transcription factors NF-κB (such as p65) directs osteoclast-specific gene expression leading to bone resorption. The marker genes whose expression are induced in osteoclast activation include tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK), which are both involved in osteoclast differentiation and facilitation of osteoclast specialized function (Boyle et al., 2003; Troen, 2004; Wada et al., 2006). In addition, an upregulation of NFAT (nuclear factor of activated T cells), a calcineurin-dependent transcription factor, plays an essential role (Ikeda et al., 2004; Komarova et al., 2005). Genetic deficiency of either NF-κB signaling components or NFAT family members has resulted in osteopetrosis owing to a block in osteoclast activation (Wada et al., 2006).

Although the effect of crocin has not been directly tested in osteoclast, some of its well-established anti-inflammatory activity may be attributed to NF-κB downregulation. For instance, crocin was found to significantly inhibit the nuclear translocation of the NF-κB p50 and p65 subunits in lipopolysaccharide (LPS)-treated RAW 264.7 cells-a murine macrophage-like cell line, leading to reduced production of inflammatory mediators (Xu et al., 2009). Pretreatment with crocin showed an inhibitory effect on NF-κB activation in TNF-α (Tumor necrosis factor-α)/IFN-γ (Interferon-γ)-stimulated keratinocytes (Park et al., 2015). Based on these findings, we propose to examine the potential actions of crocin on osteoclast cells. RANKL-induced osteoclastogenesis from murine bone marrow-derived macrophages (BMMs) has been examined in the absence or presence of crocin, and bone resorption activity of mature osteoclast treated by crocin was further investigated.

2. Methods & materials

2.1. Culture of bone marrow–derived macrophage and osteoclastogenesis

Macrophage/monocyte progenitors were isolated from mouse bone marrow and differentiated *in vitro* by M-CSF. In brief, 8-week old C57BL/6 mice were sacrificed and bone marrow cells were isolated from the femur and tibia at both legs. Bone marrow macrophages (BMMs) are then differentiated *in vitro* in α-MEM containing 10% fetal basal serum (FBS, Gibco, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 30 ng/ml M-CSF (R&D systems, Minneapolis, MN, USA). The purity of the BMMs was higher than 95% in this study. For osteoclastogenesis, RANKL (100 ng/ml, R&D systems) was also used. Cell media was changed every other day by changing half of the supernatants with fresh media containing cytokines. Crocin (Sigma, St. Louis, MO, at 0 to 200 μM) was added in the culture media in the presence of cytokines. The mouse handling procedures performed in the study have been in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee of Daqing Oilfield General Hospital.

2.2. Cell viability assays

The viability of BMM following crocin treatments was assessed by MTT (Methylthiazolyldiphenyl-tetrazolium) or LDH (lactate dehydrogenase) assay kits (Promega, Madison, WI, USA). For MTT assay, cultured cells after treatments were incubated with a premixed dye solution, and metabolically living cells were determined by the conversion of the tetrazolium component of the dye solution based on 570 nm absorbance. For LDH assay, dead cells were determined by measuring LDH, a stable cytosolic enzyme released if cell dies. After the crocin treatments, the released LDH in culture supernatants was measured by an enzymatic assay, which is based on a color change at 490 nm absorbance.

2.3. Cell apoptosis assay by TUNEL staining

Cell apoptosis is also evaluated by in situ staining of internucleosomal DNA fragmentation, which is a hallmark of apoptosis in mammalian cells. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay kits (Sigma) were used as instruction, and percentage of apoptotic cells were quantified by fluorescent microscopy.

2.4. TRAP staining

Osteoclast differentiation was measured by the TRAP (Tartrate-resistant acid phosphatase) assay. After fixed in 4%PFA (paraformaldehyde), cells were incubated with buffer containing α-naphthyl phosphate and Fast Violet B dissolved in 0.05 M sodium acetate buffer (pH = 5) with 0.05 M sodium tartrate (Sigma). Incubation was carried on for 60 min at 37 °C, and TRAP-positive cells that contained 3 or more nuclei were scored as mature osteoclast cells. The osteoclast area was also measured and analyzed by ImageJ (NIH, Bethesda, MD).

2.5. Determination of bone resorption activity

An *in vitro* bone resorption pit assay was performed to evaluate the function of mature osteoclast cell following crocin treatment. Briefly, cells after BMM differentiation were plated on bovine bone discs for overnight adherence. The cells were then incubated in the absence or presence of various concentrations of crocin (10, 50 and 100 μM) for another 48 h, followed by hematoxylin staining (resorption pits visualization). The bone slices were first treated by NaOH to remove cells, and then washed twice with PBS before staining with Mayer's hematoxylin (Sigma). The resorption area was calculated and analyzed by ImageJ.

2.6. Cytosol and nucleus fractionation

To determine the subcellular localization of NF-κB signaling mediators, subcellular fractionation was performed. Cells were first homogenized by hypotonic buffer (250 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES pH = 7.4). Nuclear was pelleted by centrifuge at 500g × 5 min, followed by wash and finally lysed in SDS sample buffer. Supernatant was saved for cytoplasm fractions.

2.7. Western blot

The protein lysates were quantitated by bicinchonininc acid (BCA) protein assay, and the same amount of total protein were run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by 1% bovine serum albumin (BSA, Sigma), and incubated with primary antibodies overnight at 4 °C. Primary antibodies include p65 (1:300), IκBα (1:200), p-IκBα (1:150) (Santa Cruz Biotechnology, Dallas, TX, USA), β-actin (1:500, Sigma), HDAC1 (1:500), CTSK (1:400), GAPDH (1:500) (Abcam, Cambridge, MA, USA), NFATc1 (1:200), and c-Fos (1:300) (Cell Signaling, Danvers, MA, USA). HRP conjugated secondary antibodies were used to visualize bands under an ECL-based imaging system. Signals were analyzed in ImageJ, and compared to controls after normalization (3 time repeats).